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Mero, Sointu

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Multiplex PCR detection of *Cryptosporidium* sp, *Giardia lamblia* and *Entamoeba histolytica* directly from dried stool samples from Guinea-Bissauan children with diarrhoea

S. Mero[1], J. Kirveskari[1], J. Antikainen[1], J. Ursing[2,3,4], L. Rombo[5,6], P-E Kofoed[3,7], A. Kantele[5,8*]

[1] *Division of Clinical Microbiology, Helsinki University Hospital, HUSLAB, Helsinki, Finland*

[2] *Department of Infectious Diseases, Danderyds Hospital, Stockholm, Sweden*

[3] *Bandim Health Project, Indepth Network, Apartado 861, Bissau, Guinea-Bissau*

[4] *Department of Microbiology, Tumour and cell biology, Karolinska Institutet, Stockholm, Sweden*

[5] *Unit of Infectious Diseases, Solna, Karolinska Institutet, Stockholm, Sweden*

[6] *Centre for Clinical Research, Sörmland County Council, Eskilstuna, Sweden and Uppsala University, Uppsala, Sweden*

[7] *Department of Paediatrics, Kolding Hospital / IRS University of Southern Denmark, Denmark*

[8] *Clinic of Infectious Diseases, University of Helsinki and Helsinki University Hospital, Helsinki, Finland*

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*Corresponding author: Professor Anu Kantele, Division of Infectious Diseases, Department of Medicine, Helsinki University Central Hospital, POB 348, FIN-00029 HUS, Finland; FAX +358-9-471 75900; tel. +358-50-309 7640; email anu.kantele@hus.fi

Authors' contributions: Study concept and design JA, JK, JU, LR, P-EK, AK; acquisition of data SM,JA,JK, JU, LR, P-EK, AK; analysis and interpretation of results SM, JA, JK, AK; drafting of manuscript SM, AK; final approval of version published SM, JA, JK, JU, LR, P-EK, AK

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Abbreviations: PCR - polymerase chain reaction; TD - travellers' diarrhoea; ATCC - American Type Culture Collection

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ABSTRACT

Background

In developing countries, diarrhoea is the most common cause of death for children under five years of age, with *Giardia lamblia, Cryptosporidium* and *Entamoeba histolytica* as the most frequent pathogenic parasites. Traditional microscopy for stool parasites has poor sensitivity and specificity, while new molecular methods may provide more accurate diagnostics. In poor regions with sample storage hampered by uncertain electricity supply, research would benefit from a method capable of analysing dried stools.

Methods

A real-time multiplex PCR method with internal inhibition control was developed for detecting *Giardia lamblia, Cryptosporidium hominis/parvum* and *Entamoeba histolytica* directly from stool specimens. Applicability to dried samples was checked by comparing with fresh ones in a small test material. Finally, the assay was applied to dried specimens collected from Guinea-Bissauan children with diarrhoea.

Results

The PCR's analytical sensitivity limit was 0.1 ng/mL for *G. lamblia* DNA, 0.01 ng/mL for *E. histolytica* DNA, and 0.1 ng/mL for *Cryptosporidium* sp. In the test material, the assay performed similarly with fresh and dried stools. Of the 52 Guinea-Bissauan samples, local microscopy revealed a parasite in 15 %, while PCR

detected 62 % positive for at least one parasite: 44 % of the dried samples had *Giardia*, 23 % *Cryptosporidium,* and 0% *E. histolytica*.

Conclusions

Our new multiplex real-time PCR for protozoa presents a sensitive method applicable to dried samples. As proof of concept, it worked well on stools collected from Guinea-Bissauan children with diarrhoea. It provides an epidemiological tool for analysing dried specimens from regions poor in resources.

Keywords : real-time PCR; *Giardia*; *Cryptosporidium*; *Entamoeba*; aetiology; developing country; children; travel; diarrhoea; parasite; stool parasite

INTRODUCTION

Diarrhoeal diseases account for 760 000 deaths annually, representing the second most frequent worldwide cause of mortality among children aged under five years, with the majority of cases in Africa and southeast Asia [1-3]. In Guinea-Bissau, West Africa, diarrhoeal diseases rank the second most common cause of death after malaria [4,5]. Research has mainly focused on bacteria and viruses as causative agents of diarrhoea in developing countries, while parasites have received less attention [6,7]. Microscopy may have led to underdiagnosis of parasites as aetiological agents [8].

Giardia and *Cryptosporidium* are common in domestic animals and some wildlife [9-11]. They both have zoonotic potential and are frequent in regions where people live in poor hygienic conditions and day-to-day contact with animals. The typical symptoms of giardiasis are watery diarrhoea, stomach cramps, and excessive gas, yet in some patients, the course of the disease is severe [6,9]. *Cryptosporidium* sp. is an important cause of moderate-to-severe diarrhoea and death among children under five years of age [6,10]. In adults, the disease is characterized by abdominal pain and watery diarrhoea, yet it may become severe and prolonged especially in immunocompromised hosts [6,10]. Of the 14 species infecting humans, *C. hominis* and *C. parvum* are reported most frequently [10-12]. The causative agent of intestinal amoebiasis, *E. histolytica,* is the only *Entamoeba* species pathogenic for humans [6]. The clinical picture of the disease varies: some cases stay asymptomatic, others may have diarrhoea and stomach ache, and a minority suffer from severe amebic dysentery with bloody stools, fever, and intense abdominal pain. It should be noted that in endemic areas asymptomatic carriage is common and, therefore, positive parasite findings per se do not directly prove causality [13,14].

Microscopic examination of stool samples has been the gold standard for diagnosis of many intestinal protozoas. The method is time-consuming, however, and requires skilled personnel, and specificity and sensitivity may thus remain unsatisfactory [6,8]. Since the last decade, molecular methods have provided

95 accurate diagnostics for numerous infectious diseases, including parasitic infections [12,15]. PCR-based
96 methods have been developed for detection of *Giardia, Cryptosporidium* and *Entamoeba* infections in
97 faecal samples [12,15-17].
98
99 To allow direct analysis of protozoa from stool samples, we developed a *Giardia lamblia, Cryptosporidium*
100 *hominis/parvum* and *Entamoeba histolytica*-specific multiplex real-time PCR method with an internal
101 inhibition control which helps to recognize the influence of various PCR inhibitors in faecal samples [12,15].
102 As studies in developing countries often suffer from unreliable supply of electricity, sample storage may be
103 hampered. Therefore we explored how well the assay performs on samples dried on filter papers. As proof
104 of concept we finally applied the assay to dried stool samples collected in Guinea-Bissau from children with
105 diarrhoea.
106

MATERIAL AND METHODS

The study protocol consists of two parts (Figure 1); firstly, the development and validation of a real-time PCR method and, secondly, application of the method to samples collected from Guinea-Bissau.

Compliance with ethical standards

Funding

The work was supported by the Finnish Governmental Subsidy for Health Science Research and by the SSAC Foundation. The funding sources had no involvement in study design, data collection, analysis, interpretation of data, writing of the report, and the decision to submit the article for publication.

Conflict of interest

All authors declare no conflicts of interest

Ethical approval

The study protocol was approved by the ethics committee of the Helsinki University Hospital, and Comité Nacional de Ética na Saúde, Instituto Nacional de Saúde Pública, Guinea-Bissau (No: 031/CNES/2010).

Informed consent

Written Informed consent was obtained from parents of each volunteering child.

Development of PCR

Study design

The PCR was designed to identify species-specific genes and universal gene regions with the internal inhibition control. The primers and probes were designed with Allele ID (Palo Alto, CA) to recognize correct

target genes (Table 1). The method was first validated with ATCC (American Type Culture Collection) genomic DNA and intestinal bacterial strains and a variety of intestinal protozoa to exclude cross-reactivity (Table 2). Preselected stool samples positive and negative for a variety of intestinal bacteria and protozoa were analysed by PCR and reference methods from routine diagnostics. As reference method for *Giardia* we used microscopy, and for *Cryptosporidium* and *E. histolytica* antigen assays.

Specificity of PCR assay tested with genomic DNA and control strains

Control genomic DNA from ATCC from each of the parasitic pathogens, *Giardia lamblia, Entamoeba histolytica* and *Cryptosporidium parvum,* were used as positive controls in the PCR (Table 2). A selection of different parasites and intestinal bacterial strains was applied as negative control to exclude possible cross-reactivity. The 15 parasites and 18 bacteria selected for this analysis are given in Table 2. For PCR analyses, stool samples positive for parasites and bacteria were collected in 100 µl Tris-EDTA buffer and DNA was purified by the easyMAG platform as described below; the supernatant (0.5 µl) was used in PCR.

Analytical sensitivity of PCR

To analyse the sensitivity of the PCR, each ATCC control DNA was diluted 10-fold and analysed by PCR in 10 parallel reactions. The analytical cut-off value was the lowest dilution where at least 9 of 10 parallel reactions were amplified in PCR. The statistical analysis was performed with SPSS 23 (IBM SPSS Statistics).

Pre-selected positive clinical samples

Pre-selected clinical control stool samples used as reference were obtained from the routine diagnostics of the parasitology laboratory of HUSLAB (Helsinki University Hospital Laboratory) after analysis by

160 microscopy (reference method for *Giardia*) and antigen detection (for *Cryptosporidium* and *Entamoeba*)
161 (ProSpecT™ *Giardia*, and ProSpecT™ *Cryptosporidium*, REMEL, United States; *Entamoeba* Celisa Path,
162 Cellabs, Australia). The samples included 26 positive specimens and 48 negative for three parasites by
163 microscopy and/or antigen detection (Table 3). No clinical data were available from these control samples.
164
165 *DNA isolation*
166
167 Total DNA was extracted from the stool samples with the NucliSENS kit (Durham, NC) using the easyMAG
168 automatic nucleic acid purification platform (bioMérieux, Marcy l´Etoile, France), as described by the
169 manufacturer. Briefly, the stool samples were suspended to Tris-EDTA buffer and 100 µl added to lysis
170 buffer. The extraction was performed by the general method of easyMAG platform and, at the end, eluted
171 to a volume of 55 µl. Formalin-fixed samples (500 µl) were centrifuged 12.000 g for 1 minute, the
172 supernatant was pipetted off and 500 µl Tris-EDTA buffer was added. The suspension was used for DNA
173 extraction as described earlier.
174 Sample pretreatment with Precellys® 24 (Bertin Techonologies, France) high-throughput tissue
175 homogenizer was tried out with 17 (of the total 74) samples (3 *Giardia*, 5 *Cryptosporidium*, 3 *Entamoeba*
176 *histolytica*, 6 negative) before the DNA isolation to ensure successful DNA extraction. Briefly, the stool
177 samples were suspended to Tris-EDTA buffer in Precellys tube with ceramic beads, and run 6.500 rpm 2x 35
178 seconds homogenization protocol, as described by the manufacturer. The homogenized (n = 17) and non-
179 homogenized stool suspensions (100 µl) were used for DNA extraction and PCR results were compared to
180 each other.
181
182 *PCR amplification*
183
184 The multiplex-PCR was performed on the Mx3005P detection system (Agilent Technologies, Inc., CA). The
185 thermocycling was performed in the following conditions: initial denaturation at 95°C for 15 minutes, 45

cycles of denaturation at 94°C for 1 minute, and annealing/extension at 60°C for 1 minute. The 25 µl reaction contained 1x Multitect NoROX master mix (Qiagen, Venlo, Netherlands), 1.25 µl primer and probe mix (Table 1) and 5 µl template DNA. Positive samples with three and negative samples with two parallel reactions were analysed with the PCR. Interpretation as positive required a positive result in at least two of three reactions.

Potential inhibition of PCR reaction by faecal constituents was identified with help of an internal inhibition control, *Oryza sativa* terminal flower gene [18].

Analysis of stool samples collected in Guinea-Bissau

Performance of dried samples in the PCR assay

Before starting stool collections in Guinea-Bissau, the performance of the PCR method with dried samples was tested by comparing 28 positive control specimens analysed with both approaches, after drying vs. fresh specimens. The controls were routine samples found positive for these parasites at the HUSLAB routine laboratory (15 *Giardia*, 11 *Cryptosporidium* and two *E. histolytica*). A part of each specimen was subjected to DNA was extraction directly from the fresh preparate, while another part of the same sample was wiped onto filter paper, then left to dry for one (10 samples) or two weeks (18 samples) in room temperature. After drying, the spot with the stool was cut out from the filter paper and moisturized within NucliSENS lysis buffer (Durham, NC) overnight at room temperature. After powerful vortexing, the filter papers were removed from the buffer and the remaining liquid was used for the analyses. DNA extraction was carried out with easyMAG platform and multiplex PCR-analysis performed as described above.

Patient samples

A total of 52 children with diarrhoea were recruited between April and August 2011 among consecutive patients seeking medical care at the Bandim Health Centre in Guinea-Bissau. These patients represented a sub-population of another study for which stool samples were collected during 2010-2012. At the laboratory of the health centre, all faecal samples were examined microscopically and stool was wiped on a filter paper (Hemoccult®, Becman Coulter, Ireland) and left to dry for 24 hours. Further analyses of the dried samples were performed in HUSLAB, as described above.

RESULTS

Development of PCR

The multiplex PCR method was designed to identify species-specific genes and universal gene regions of *Giardia lamblia, Cryptosporidium* sp. and *Entamoeba histolytica.* The assay showed no cross-reactivity with other parasites or stool bacteria (Table 2). By contrast, it proved positive for each of the three validated parasites obtained from ATCC (*Giardia*, *Cryptosporidium* and *Entamoeba*; Table 2).
No inhibition was detected with internal inhibition control in any of the control samples (Table 1).

Sample pretreatment with Precellys® 24 high-throughput tissue homogenizer was tried out for 17 samples before DNA isolation. All three samples positive for *Giardia* proved negative by PCR after pretreatment with homogenization. Five samples were found positive for *Cryptosporidium*s by PCR (Ct 40.66) when analysed without homogenization; only four of them remained positive (Ct 38.13) after homogenization. Three samples with *E. histolytica* proved positive both without (Ct 29.37) and with (Ct 27.32) prior homogenization. Further analyses were carried out without homogenization. The specificity of the PCR was analysed directly from stool samples using preselected clinical material from routine diagnostics (Table 3). Of the total of 48 preselected specimens negative by reference methods, 46 were found negative also when using PCR. In the two remaining samples *Giardia* was detected positive by PCR and negative by reference methods. Of the 26 preselected stool samples positive when employing reference methods, 23 were positive by PCR. The five positive by reference methods but negative by PCR included one *Giardia* and four *Cryptosporidium* cases as described below in detail.

Giardia. G. lamblia was found by PCR in 10 of 11 samples initially tested positive for *Giardia* by the reference method (microscopy). The single specimen negative by PCR showed only very few parasites in microscopic examination.

Cryptosporidium. Of the 11 samples positive for *Cryptosporidium* sp. by the reference method (antigen detection), seven were confirmed by PCR. Of the four PCR negative samples, one amplified in only one of three parallel reactions and was thus decoded as negative.

The analytical sensitivity of the PCR was determined by 10-fold dilutions of the control DNA. The detection limit of the PCR was 0.1 ng/mL (8000 genome copies/ml) for *G. lamblia* DNA, 0.01 ng/mL (450 genome copies/ml) for *E. histolytica* DNA and 0.1 ng/mL (10 000 genome copies/ml) for *Cryptosporidium* sp. DNA. The validation experiments showed that the assay had 82.1 % overall sensitivity (*Giardia* 91 %, *E. histolytica* 100 % and *Cryptosporidium* 64 %) and 95.8 % specificity with 92.0 % positive and 90.2 % negative predictive value and no significant statistical differences between reference methods and developed PCR.

The performance of the PCR method in the analysis of dried faecal samples was evaluated with help of 28 control stool specimens each positive for one or more of the parasites included. These were individually analysed as fresh samples and compared to results obtained after drying on filter paper. The assay performed equally well with both: on the average, dried samples showed Ct values 2.3 lower than the fresh ones.

Results of Guinea-Bissauan samples

A total of 52 children were enrolled during the study period; demographics and clinical data are shown in Table 4. PCR analyses revealed *Giardia* in 44 %, *Cryptosporidium* in 23 %, and *E. histolytica* in 0 % of the stools collected (Table 5.) Five (10 %) cases with mixed infection of *Giardia* and *Cryptosporidium* were

recorded. Thirty-eight percent (20 of 52) of the samples proved negative for all three parasites tested. There was no evidence of amplification inhibition in any of the samples with the DNA extraction method used.

Microscopic examination carried out at Bandim Health Centre in Guinea-Bissau only detected *Giardia* in two of the 23 (9 %) samples positive by PCR (Table 5). Two that were found negative by PCR were microscopically identified as positive, one for *Giardia* and the other for *Entamoeba*. No cases of *Cryptosporidium* were detected by microscopy.

DISCUSSION

Despite its poor sensitivity and specificity, microscopy, the traditional method of examining stool parasites is still widely used in clinical laboratories in advancing and advanced economies alike [8,12]. We present a new real-time multiplex PCR method applicable to both dried and fresh stool samples. As proof of concept, the method was applied to dried specimens collected in Guinea-Bissau from children with diarrhoea. The PCR results were compared to findings obtained locally by microscopic examination.

Methodological considerations

Advantages of PCR

New molecular methods such as PCR provide sensitive and specific alternatives to microscopy for diagnosis of stool parasites. The requirement of separate PCR assays for each parasite and complex pre-analytical handling of samples may render the assays quite time-consuming. Our multiplex PCR surpasses these shortcomings: the three parasites are identified in the same multiplex directly from stool samples. Unlike microscopic analyses, the performance of the assay does not vary according to laboratory staff skills.

Parasite types covered

The PCR was designed to cover only the clinically most important ones of the three parasites' several species. Of *G. lamblia's* seven genetically distinct genotypes the assay was designed to cover the two species known to infect humans [9,12]. Similarly, *Cryptosporidium* sp. constitutes a wide and heterogeneous group with 30 species at least 14 of which have been reported to infect humans [10,11]. The assay was devised only to cover the two most frequent causes of human infection, *Cryptosporidium*

303 *hominis* and *C. parvum*, the other species infecting humans (e.g. *C. canis, C. felis* and *C. meleagridis*) being
304 of minor relevance to public health [11,19]. For *Entamoeba*, PCR offers a valuable approach to distinguish
305 pathogenic *E. histolytica* from nonpathogenic *E. dispar* [6] – a substantial improvement to routine
306 diagnostics, as this distinction cannot be achieved by microscopy [6,12,20].
307
308 *Sampling on filter paper*
309
310 Filter paper has become increasingly popular in collecting, transporting and storing varied specimens from
311 humans, animals and plants with analyses of a diverse range of biochemical and serological assays
312 undertaken even years later [21-23]. The use of filter paper for parasitological stool analyses has so far only
313 been described in a few reports [23,24]. Our data shows filter paper to work well with stool samples, a
314 great advantage in trials and epidemiological studies in regions where unreliable supply of electricity may
315 pose a major obstacle to research.
316
317 *Sensitivity and specificity of assay*
318
319 The assay showed 82.1 % overall sensitivity and 95.8 % specificity, both calculated by comparing PCR
320 findings with reference methods, microscopy results for *Giardia* and antigen tests for *E.*
321 *histolytica* and *Cryptosporidium*. In regard to the small number of samples, comparison with gold standards
322 for various parasites, and the PCR's inability to detect all species, the figures should be considered as
323 approximate, but nevertheless valid proof of concept.
324 In a more detailed analysis the assay was found more sensitive for *Giardia* 91 % (10/11 cases identified) and
325 *Entamoeba* 100 % (3/3) than *Cryptosporidium* 64 % (7/11). The low sensitivity figures of *Cryptosporidium*
326 may have several explanations: reference tests (antigen detection) may have given false positive results
327 (inadequate specificity of antigen assay) or remained positive even if no live organisms were present. Our
328 negative PCR findings may thus only indicate the absence of live protozoa: free DNA is not likely to resist

329 active DNAses during passage through the gastrointestinal tract [25,26]. Furthermore, the enzyme
330 immunoassays (EIA) may have covered more species if they use an antigen common to the whole genus.
331 Sample pretreatment with tissue homogenizer appeared not to increase sensitivity, contrary to the findings
332 by Mary et al. [27]. However, our comparison between homogenized and nonhomogenized samples may
333 not have been fully valid, as we used frozen samples, and the freezing process may already have broken the
334 targets (oocysts of *Cryptosporidium* and cysts of *Entamoeba*).
335 As for specificity, the assay seemed to perform well, not identifying any of the bacterial stool pathogens – a
336 prerequisite for this type of assay, since bacterial pathogens are very common causes of diarrhoea.
337
338 *Performance of assay in analysing Guinea-Bissauan samples*
339
340 Our PCR seemed to perform well with the Guinea-Bissauan samples, detecting all but one (possibly *E.*
341 *dispar)* of the parasites identified by local microscopy. The number of cases not identified by microscopy
342 but yielding positive results by PCR proved surprisingly high (8 vs. 32 samples). This difference between
343 performance with clinical and validation samples may be explained by factors affecting the accuracy of
344 microscopy: laboratory facilities, quality of microscopes, and time spent on each specimen. All our
345 validation samples were studied in a well equiped modern facility by a highly skilled technician. The local
346 microscopic examination revealed *Giardia* in only 28 % of the cases identified positive by PCR. As for
347 *Cryptosporidium,* 20 of 32 samples proved positive by PCR, while no pathogens were identified by
348 microscopy. However, the two results should not be compared, since to be visualized, *Cryptosporidium*
349 requires a specific staining not available at the laboratory in Guinea-Bissau. Previous studies have reported
350 greater sensitivity for PCR than microscopy [16,28-31].
351
352 *Clinical considerations of Guinea-Bissauan cohort's results*
353

The clinical samples revealed a high frequency of intestinal parasites in children with diarrhoea. The proportion of samples positive for at least one parasite was 15 % with the local microscopy (*Giardia* 6 %, *Entamoeba* 1 %, other parasites 8 %) and 62 % with our PCR (*Giardia* 44 %, *Cryptosporidium* 23 %). The PCR results differ from those reported for East African children where *Cryptosporidium* has proved more frequent than *Giardia* [17,32,33]. The high prevalence of *Giardia* may reflect a regional difference, for the prevalence rates reported both by Ferreira et al. in Guinea-Bissauan children (46 % by microscopy, 56 % by PCR) [24] and, the same year, by Ignatius et al (19.8 % by microscopy, 60.1 % by PCR) in Rwandan children [34] resembled our findings. In many investigations, the prevalence may have been underestimated, if merely using microscopy [16].

In accord with the GEMS study in sub-Saharan African and South Asian children, we found no cases with *E. histolytica* [14]. The prevalence of *E. histolytica/dispar* has been reported to increase with age [17,32]; all our subjects were younger than five.

The patients with Giardia (91%) and Cryptosporidium (100%) both typically had a watery diarrhoea. The symptoms had lasted 3.3 days vs 2.7 days (median) and included on the average 4.5 vs 5.7 stools per day, respectively. The data suggest that children with *Cryptosporidium* were admitted earlier because they had more vigorous symptoms.

Limitations

While our PCR method only recognizes three intestinal parasites, some other potentially relevant species were left uncovered. Among these is *Dientamoeba fragilis* [35-37]. Of *Cryptosporidium* sp. our assay only identifies *C. hominis*/*parvum*, failing to cover some other *Cryptosporidium* species of potential importance for immunocompromised (e.g. HIV-positive) patients [10,19]. The moderate sensitivity of the *Cryptosporidium* assay may partly be overcome by traditional multiple sampling.

The limitations of the Guinea-Bissauan data include low number of specimens and lack of asymptomatic children as controls, and absence of simultaneous analyses that would cover bacterial pathogens and viruses commonly causing diarrhoea in developing countries [6]. Future studies overcoming these limitations are warranted to enable evaluation of each pathogen's role in causing the symptoms – in our current data part of the parasites may simply reflect carriage of pathogen.

Conclusion

We introduce a high-throughput multiplex PCR method for identifying three common intestinal parasites, *Giardia lamblia, Cryptosporidium* sp. and *Entamoeba histolytica*. The assay can be used for direct analysis of dried stool samples: as proof of concept, this approach proved applicable to dried samples collected from a region poor in resources. Keeping in mind that positive parasite findings in endemic areas do not directly prove causality, the present data from Guinea-Bissau call for a larger investigation comparing findings between asymptomatic children and those with diarrhoea. Our approach using dried specimen offers a practical tool for epidemiological studies in developing regions.

395

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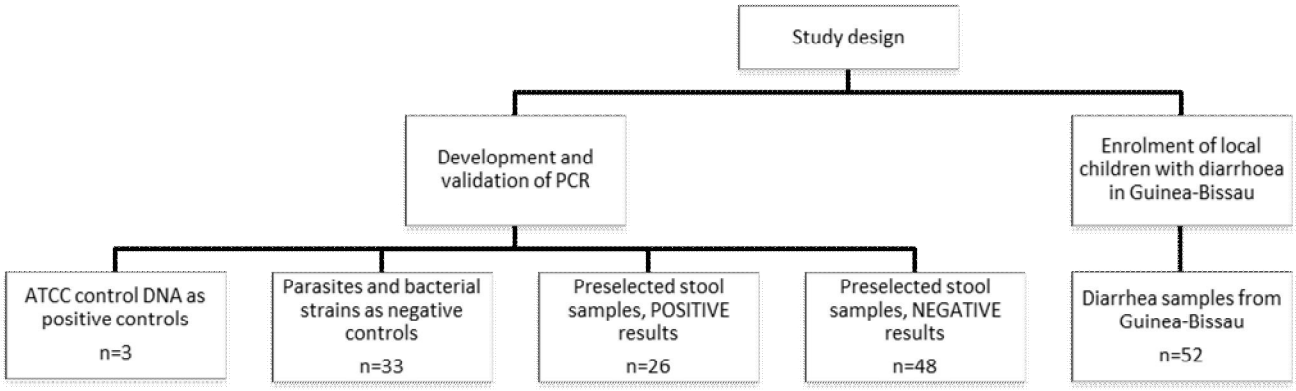
481

482

483 TABLES AND FIGURES



484

485 Figure 1. The study protocol consist of two parts; first, development and validation of PCR method, and

486 second, applying it to samples collected in Guinea-Bissau.

487

488 Table 1. Primers and probes of multiplex PCR

489

Target organism and oligonucleotide	Gene	Concentration (µM)	Oligonucleotide sequence (5´→3´)
Giardia lamblia			
F_giard_03	*18S*	0.2	TTCCGGTCGATCCTGCC
R_giard_03	*18S*	0.2	GTTGTCCTGAGCCGTCC
P_giard_03	*18S*	0.2	56-FAM/ACGAAGCCATGCATGCCCGCT/3IABkFQ
Entamoeba histolytica			
F_ehis_02	*18S*	0.1	AGACGATCCAGTTTGTATTAG
R_ehis_02	*18S*	0.1	GGCATCCTAACTCACTTAG
P_ehis_02	*18S*	0.1	JOEN/ACAAAATGGCCAATTCATTCAATGAA/3IABkFQ
Cryptosporidium sp.			
F_cowp_01	*cowp*	0.2	TCTGGAAAACAATGTGTTC
R_cowp_01	*cowp*	0.2	GGCATGTCGATTCTAATTC
P_cowp_01	*cowp*	0.2	5TexRd-XN/CCTCCTAATCCAGAATGTCCTCCAG/3IAbRQSp
Internal control *			
F_ory	*ory*	0.1	CTAATCCCAGCAACCCAACC
R_ory	*ory*	0.1	CTAATCAATGTGAGACATATGATAGAAATC
P_ory	*ory*	0.1	Cy5/cctGcaCtgGtaAgctatg/Iowa Black RQ **
Template DNA for internal control			
ory_fw		2^{-10}	TGCTCCTAATCCCAGCAACCCAACCTTGAGGGAATACCTGCACTGGTAAGCTATGCT CTTGCAATTGTTGTGATTTCTATCATATGTCTCACATTGATTAGTGATCTA
ory_rv		2^{-10}	TAGATCACTAATCAATGTGAGACATATGATAGAAATCACAACAATTGCAAGAGCAT AGCTTACCAGTGCAGGTATTCCCTCAAGGTTGGGTTGCTGGGATTAGGAGCA

490 * [18]

491 ** small caps: normal nucleotide; large caps: LNA modified nucleotide

492

493 Table 2. Parasites and intestinal bacterial strains serving as negative controls and genomic DNA from

494 ATCC as positive controls in PCR validation.

495

Negative parasite controls (n=15)	Result in PCR	Negative bacterial controls (n=18)	Result in PCR
Ancylostoma duodenale	negative	*Lactobacillus* sp.	negative
Ascaris lumricoides	negative	*Proteus vulgaris*	negative
Blastocystis hominis	negative	*Pseudomonas aeruginosa*	negative
Chilomastix mesnili	negative	*Salmonella enteritidis*	negative
Cyclospora cayetanensis	negative	*Citrobacter freundii*	negative
Dientamoeba fragilis	negative	*Staphylococcus aureus*	negative
Echinococcus multilocularis	negative	*Clostridium perfringens*	negative
Endolimax nana	negative	*Klebsiella pneumoniae*	negative
Entamoeba coli	negative	*Acinetobacter baumannii*	negative
Entamoeba hartmannii	negative	*Enterococcus faecalis*	negative
Iodamoeba butschilii	negative	*Yersinia enterocolitica* 0:3	negative
Malaria falciparum	negative	*Bacteroides fragilis* group	negative
Taenia saginata	negative	*Enterobacter cloacae*	negative
Trichuris trichiura	negative	*Escherichia coli*	negative
Trypanosoma cruzi	negative	*Streptococcus constellatus*	negative
		Fusobacterium nucleatum	negative
		Prevotella buccae	negative
		Campylobacter jejuni	negative
Positive DNA controls (n=3)			
Giardia lamblia ATCC-50803D*	positive		
Entamoeba histolytica ATCC-30459D*	positive		
Cryptosporidium parvum ATCC-PRA-67D*	positive		

496 *Genomic DNA

497

Table 3. Pre-selected clinical stool samples. As reference method for *Giardia*, we used microscopy, and for *Cryptosporidium* and *E. histolytica* antigen assays.

	Reference methods	PCR * n (Ct-values, range)
Negative samples	48 **	46 [1]
Positive samples	26	23
Giardia lamblia	11	10 [2] (32.00-40.22)
Cryptosporidium sp.	11	7 [3,4,5,6] (28.26-42.38)
Entamoeba histolytica	3	3 [7] (24.83-33.74)

* Internal and inhibition control was amplified of all samples.

** Twelve samples were positive for other parasites by microscopic examination (4 *Blastocystis hominis*, 7 *Dientamoeba fragilis*, 1 *Endolimax nana*).

[1)] Two samples were *Giardia* positive by PCR (Ct 35.66 and 39.54), but the result could not be confirmed with the reference method.

[2)] One *Giardia* sample positive by microscopy proved negative by PCR; microscopic examination showed very few parasites.

[3)] One sample positive for *Cryptosporidiu*m with reference methods was amplified in only 1 of 3 parallel reactions by PCR (Ct 38.18) and thus considered negative.

[4)] One sample positive for *Cryptosporidium* with reference methods was amplified in 2 of 3 parallel reactions by PCR (Ct 38.43 and 41.62) and thus considered positive.

[5)] Two samples showed very few *Cryptosporidium* parasites by microscopy and yielded negative results by PCR.

[6)] One sample was positive by both reference methods, while PCR was negative.

[7)] One sample found *Giardia lamblia* as a second parasite.

517 Table 4. Demographics of 52 Guinea-Bissauan children with diarrhoea

518

	All subjects n=52 (% of all)	
Background information		
Female	20	(38)
Male	32	(62)
Median age, y (range) [1]	1.5	(0-5)
Diarrhoea		
Stools per day, mean (range)	5	(2-10)
Duration in days, mean (range)	3	(1-14)
Visible mucus	35	(67)
Bloody	5	(10)
Watery	47	(90)
Other illnesses or symptoms		
Stomach pain	13	(25)
Vomiting	14	(27)
Fever	37	(71)
Influenza	14	(27)
Malaria	2	(4)
Other	1	(2)

519 [1)] information missing from two children

520

521 Table 5. Results of 52 Guinea-Bissauan children's diarrhoea stool samples.

522

	Diarrhoea samples n=52 (%)		
	PCR	Ct value, range	Microscopy
Negative samples	20 (38)		44 (85)
Positive samples	32 (62) [1]		8 (15) [2]
Giardia lamblia	23 (44)	(20.49-31.91)	3 (6) [3]
Cryptosporidium sp.	12 (23)	(25.65-37.12)	NA
Entamoeba histolytica	0 (0)		1 (2)

523 [1)] five mixed infections by *Giardia* and *Cryptosporidium*

524 [2)] 4 specimen were positive for other parasites (2 *Entamoeba coli*, 1 *Endolimax nana*, 1 *Ancylostoma* sp.)

525 but the PCR was negative

526 [3)] 1 of the 3 *Giardia* findings by microscopic was negative by PCR

527 NA, not applicable as no staining was performed in Guinea-Bissau